Influence of Bromobenzene on the Induction of Skin Tumors by 3,4-Benzpyrene

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It has previously been demonstrated that the rate of tumor induction in mice by chemical carcinogens of the hydrocarbon type is retarded by a series of compounds containing labile halogen atoms (9, 10). These halogen compounds ranged from chloro-acetal, a compound of relatively low reactivity, to a variety of highly active acid halides. The velocity constants of their reaction with cysteine under physiological conditions and their power of checking cell glycolysis followed the same order and ran parallel with their capacities for retarding tumor induction.

The question arose whether the inhibition of the glycolytic process was the effective determinant of the slowing of the carcinogenic process, since, in addition to this action, all the compounds used could affect other cell mechanisms by fixation of sulphydryl groups. This more generalized concept, that disturbances of sulphur metabolism interfere with carcinogenic action, seemed worthy of investigation. For this purpose, substances were required that would affect sulphur metabolism but be devoid of direct, specific action on the glycolytic mechanism. Compounds eliminated as mercapturates should fulfil this role, provided that detoxication occurred locally in the skin. The literature dealing with the elimination of bromobenzene and allied substances describes the liver as the principal site of detoxication, since the toxic substances were normally administered via the stomach. Evidence will be given that bromobenzene can also be detoxicated in the skin and that its application to an area of skin under the influence of a carcinogen effectively retards, and sometimes prevents, the completion of the carcinogenic process.

Since the glycolysis-checking compounds with their active halogen atoms, and bromobenzene with a halogen atom unreactive in the same sense, have the common property of disturbing sulphur metabolism, the inference is made that the latter is the biochemical basis of their anticarcinogenic action.

* Because of the difficulties of international communication the author has not read proof of this article.

EXPERIMENTAL

Demonstration of inhibition of carcinogenic process by bromobenzene.—Batches of 30 mice were used in each experiment. The mice were not of pure strain but were all descendants of some 150 hybrids from two breeders’ stocks. Their diet was uniform, consisting solely of a mixture compounded and pressed into small bricks and obtained commercially. No details of its composition are given, but its well-balanced nature is reflected in the general good health and normal growth of the mice. From 24 to 28 mice always survived in any treated batch, even if the experiment lasted for 6 to 8 months.

3,4-benzpyrene dissolved in ether containing 2 per cent of liquid paraffin was applied in 0.3, 0.1, or 0.03 per cent concentration twice weekly (Mondays and Thursdays) with a No. 4 brush in the scapular region over an area of approximately 1 sq. cm. Fifteen per cent bromobenzene, by volume, in ether was applied 4 times weekly (Tuesdays, Wednesdays, Fridays, and Saturdays) with a No. 6 brush over the benzpyrene-treated area but widely overlapping it. This amounted to roughly 90 mgm. of bromobenzene per mouse per week. This dose of bromobenzene had no measurable effect on the weight of the mice, and no impairment of health occurred over the longest experimental period.

The results are shown in Fig. la, b, and c for benzpyrene concentrations of 0.3, 0.1, and 0.03 per cent respectively. Duplicate experiments were made in some cases and the curves show how closely the average induction times corresponded in such pairs. When the weaker concentrations of benzpyrene were used, almost all the surviving control mice, treated with benzpyrene alone, carried tumors, many of which were epitheliomatous, before a single wart was visible in the bromobenzene-treated mice.

Smaller amounts of bromobenzene caused proportionately less inhibition. To avoid gross systemic effects on the mice due to irreplaceable sulphur losses, larger amounts of bromobenzene were not used. Since it has been shown (3, 12) that benzpyrene or a fluorescent...
derivative remains at the site of its application for several days, it is conceivable that the observed inhibitory effects of bromobenzene could be attributed to the ether used for its daily application. Dispersal of the carcinogen or its active derivative might occur, the progressive dilution lowering its effective concentration.

That the retarding effect of bromobenzene on tumor induction is due to its local rather than systemic action is clearly shown by submitting mice to exactly the same amounts of benzpyrene and bromobenzene, the former applied as usual to the scapular region and the latter to an area of the lower back. In this case, tumors appeared at the normal rate, uninfluenced by whatever deviations from the general level of sulphur distribution the bromobenzene produced. It is clear that local metabolic disturbances in the skin itself are responsible for the biological result, and the presumption is that mercapturate formation takes place in this tissue.

Local sulphur depletion is suggested by another observation. When bromobenzene was applied to the nonepilated skin, it caused a slow, progressive thinning of the hair, tending towards baldness, but under the conditions used never attaining this. This potentiality was masked in the experiments demonstrating anticarcinogenic action, since loss of hair is a characteristic episode in chemical carcinogenesis. In the case of bromobenzene, where the mechanism of detoxication is understood, this effect can reasonably be attributed to a lowering of the level of available sulphur in the hair follicles, which are normally sites of active sulphur metabolism. By analogy, it might be suggested that processes involving sulphur loss are accompaniments in the biological effects of carcinogens. Though naphthalene (6) and anthracene (7) are partially excreted as mercapturates, there is as yet no direct chemical evidence that the carcinogenic or noncarcinogenic hydrocarbons with larger molecules are similarly dealt with, even to a small extent, by the body.

In this connection it may be mentioned that, of the common organic solvents used in the application of carcinogens, benzene tends to delay tumor induction more than, e.g., ether (11). The recent finding of Zbarsky and Young (19) that benzene is partially excreted as a mercapturate may, by analogy with the anticarcinogenic effect of bromobenzene, be an explanation of this hitherto puzzling phenomenon.

**Action of Bromobenzene on Precancerous Mouse Skin**

The word “precancerous” has no clear connotation in terms of biochemistry. Multiple studies on the metabolism of formed cancer cells and their normal prototypes have demonstrated certain broad differences of enzymic equipment, always of a quantitative type, that accompany, or conceivably cause, the carcinogenic change. But not even the most diffuse picture of metabolic happenings during the “latent period” has emerged. One point, however, seems clear. The early responses of the cell to carcinogens are reversible.

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**Fig. 1.—Retarding effect of bromobenzene on the rate of tumor induction by 3,4-benzpyrene applied at different concentrations.**

*a. 0.3% 3,4-benzpyrene. Two separate experiments.*

*b. 0.1% 3,4-benzpyrene. Two separate groups of mice treated with bromobenzene.*

*c. 0.03% 3,4-benzpyrene.*
within limits not accurately definable. No evidence is at hand to judge between the alternative conceptions that the carcinogenic change is a sudden event or represents the culmination of a gradual series of changes from normality. In either case, by choosing a point in time prior to the emergence of visible warts and inducing opposing reactions by chemical means, the possibility is opened up of using potential reversibility as the basis of a preventive chemotherapy.

As an extension of the experiments above demonstrating inhibition of tumor induction, the effect of a local depletion of sulphur in a precancerous skin was studied. Several experiments, differing only in the degree of pretreatment with carcinogen, have yielded essentially the same result (Fig. 2). In this case 0.3 per cent benzpyrene was applied in the usual way for 11 weeks to two batches of 30 mice each and then discontinued. From this point one group of mice received bromobenzene treatment, the second group serving as controls. Twenty per cent bromobenzene in ether was painted widely over the benzpyrene-treated area of both tumor-bearing and tumor-free mice with a No. 6 brush 4 times weekly. After 26 weeks no further bromobenzene was applied and the mice were examined at intervals. Fig. 2 shows the course of the experiment up to the 26th week. Between the 26th and 39th weeks 3 further warts appeared in the bromobenzene-treated group, but the remaining 9 mice of this group remained free of tumors after a year. Rather a high percentage of mice in both groups carried tumors at the 11th week, and the course of these established tumors was not visibly affected by the bromobenzene.

It is clear that the bromobenzene causes a pronounced delay in the later tumor incidence, and in many cases entirely prevents the emergence of tumors. When smaller amounts of benzpyrene were used, say over 6 to 8 weeks, a similar delaying effect was observed, but in these cases the effect was not so obvious since warts only appeared over a very extended time even in the controls.

The conclusion is reached that intermittent interference with the sulphur metabolism of skin in a precancerous condition can delay, and often prevent, the normal course of carcinogenesis. The biochemical basis of this effect is assumed to be the reversibility of reactions involving sulphur that occur in the "latent period."

**Effect on Growth Changes and Sulphur Distribution in the Urine of Mice Treated with Bromobenzene via the Skin**

In the experiments where 15 per cent bromobenzene was applied 4 times weekly to adult mice no deleterious effect was observed; the records of weight changes over several months showed no significant deviation from those of untreated controls. Evidently the organism readily compensates through normal food intake for the temporary sulphur losses produced by this degree of detoxication. But by using younger mice, and applying 50 per cent bromobenzene in ether 4 times a week, growth retardation was readily demonstrated and its correction, by stopping the application of bromobenzene, followed in the usual manner.

Parallel results were obtained in a study of the urinary sulphur distribution. An examination of the urine of mice used in the experiments demonstrating the anticarcinogenic action of bromobenzene failed to show consistent changes that were outside normal fluctuations. When a similar amount of bromobenzene was incorporated in the diet, the same uncertainty of significant changes was found. Again, by increasing the degree of detoxication through the application of 50 per cent bromobenzene, as above, to the skin, notable changes in urinary sulphur distribution, shown graphically in Fig. 3, were obtained. By the method of McGuinn and Sherwin (13) a very small amount of \( p \)-bromophenylmercapturic acid was isolated from the urine.
Evidently bromobenzene administered via the skin route is excreted by the body in the same way as when given via the stomach. These results, of course, do not prove specifically that the skin is the site of detoxication, though the biological indications suggest this to be the case.

**Chemical Evidence that Bromobenzene is Detoxicated in the Skin**

Analysis of changes in sulphur distribution in the skin following the application of bromobenzene supports the view that this tissue is not merely a medium for transmission of bromobenzene to the liver, but itself possesses the capacity for detoxication.

Rather than estimate the total sulphur in the skin, which is probably mainly in bound form and not substantially altered by slight applications of bromobenzene, it was decided to use the reservoir of labile sulphur, mainly glutathione, as an indicator of disturbed sulphur metabolism. The literature available provided no data on the normal glutathione content of mouse skin, even though many other tissues have been studied in this respect. All the estimates reported here were made on whole skin, since the technics for separating epithelium used, e.g., by Baumberger, Suntzeff, and Cowdry (2) would adversely affect this labile constituent. Each estimation necessitated the use of 5 to 10 mice to obtain consistent results. For the quantitative determinations mice were epilated over the whole back 2 days before using. The hairless skins were removed after death and quickly minced twice in a fine mincer. The weighed mince was ground with several times its weight of clean silver sand in a mortar, 20 cc. of 10 per cent sulphosalicylic acid solution added, and the mixture reground. After 10 minutes' standing this was filtered at the pump and gave a clear filtrate. The sand-mince was reground with 10 cc. of 10 per cent sulphosalicylic acid solution, allowed to stand for 5 minutes and again filtered. Aliquots of the combined filtrates were titrated against (a) \( \frac{N}{1000} \) I\(_2\), (b) \( \frac{N}{1000} \) KIO\(_3\), and (c) 0.01 per cent phenol-indo-2,6-dichlorophenol. (a) and (b) should give identical figures representing the sum of the glutathione and ascorbic acid contents, and (c) the ascorbic acid content. The end point when KIO\(_3\) was used was occasionally vague, though Woodward and Fry (18), using it for the estimation of the glutathione content of blood, found a sharp end point. The extract contained no detectable protein, but sometimes was slightly opalescent, and this may have been responsible for the uncertain end point. In the results presented here only the values obtained by iodine titration are given. On the basis of iodine equivalents 1 mgm. of ascorbic acid corresponds to 3.38 mgm. of glutathione, and the glutathione content was obtained by deducting (c) from (a). The averages of many of these determinations on normal skins are shown on the left of Fig. 4. The variations were rarely greater than ±5 per cent when mice of about the same age were used. The values for glutathione and ascorbic acid tended to fall with increasing age of the mice.

The fall in glutathione content after applying bromobenzene to the skin was first shown qualitatively by the use of sodium nitroprusside. To assess the effect of bromobenzene quantitatively, the whole backs of a group of 5 to 10 mice were painted with a suitable dilution 4 times at half-hourly intervals. The glutathione and ascorbic acid estimations were then made at varying intervals after the last application. All the data of one typical experiment, in which 20 per cent bromobenzene was used, are gathered in Fig. 4.

In this experiment the amount of bromobenzene applied was 5 to 6 times greater than the daily application in the experiments demonstrating inhibition of carcinogenesis. Under the latter conditions a milder and possibly less prolonged disturbance of sulphur metabolism may be inferred.

The principal features that emerge from these findings are: (a) The high speed of detoxication in the skin, and the relatively rapid recovery to a normal level. (b) The concomitant fall in the ascorbic acid content of the skin that occurs with the reduction of the glutathione level, and the parallel rates of their recoveries to normal values. It may be added that successive applications of ether to the skins of mice in amounts greatly exceeding those used for applying the bromobenzene produced no detectable changes...
in the concentration levels of glutathione and ascorbic acid.

Observations on these results.—The experimental conditions were chosen arbitrarily mainly with a view to avoiding general systemic deterioration in the mice from excessive sulphur losses. The doses of bromobenzene applied were readily tolerated and general health and normal growth were maintained. The measurements of the fall and subsequent rise in the skin glutathione levels reveal the fact that this degree of treatment produces only slight local intermittent disturbances of sulphur metabolism. Yet these occasional falls in the level of the reservoir of labile sulphur are adequate to cause considerable delays in the carcinogenic action of benzyrene, and in some cases to annul it. It is conceivable that the recovery to normal glutathione concentration is not a true index of a complete return to normal sulphur metabolism, and that the disturbances in enzymic systems dependent on sulphhydryl groups for their proper functioning persist much longer than this indicator suggests. Until this is proved it must be assumed that well-spaced fluctuations in local sulphur metabolism are responsible for the biological results.

These conditions may be contrasted with the continuity of sulphur imbalance that prevails during typical experiments on the relation of diet to cancer. Examples of the effects on carcinogenesis of such prolonged impairment of normal metabolism are found in the work of Tannenbaum (14) and White and Ander- vont (15). In the latter case the occurrence of spontaneous mammary gland tumors in the females of a strain of mice with a customary incidence of 100 per cent was entirely prevented by a diet relatively low in its content of S-containing amino-acids. Extreme growth failure and disturbed ovarian hormonal function were the accompaniments of the failure in tumor occurrence, making interpretation difficult. A less drastic dietary regimen, perhaps with S-deficient diets alternating with complete diets, might permit a differentiation between any primary effect of S-deficiency on spontaneous carcinogenesis and such secondary effects as may arise from disturbed hormonal function.

Conversely, as an extension of the experiments with bromobenzene, it is interesting to speculate on the effect of enhancing local sulphur deficiency either by intermittent use of more potent inhibitors or, preferably, by promoting greater continuity of slighter disturbances, with the object of entirely opposing carcinogenic action.

Little is known of the biochemical mechanisms involved in the action of chemical carcinogens. Many studies (4, 5, 8, 12) have shown that a large but uncertain percentage of carcinogenic hydrocarbons administered intravenously or intraperitoneally are eliminated as oxidized derivatives. These oxidation products exhibit little carcinogenic activity (4) and are normally regarded as end products of detoxication processes. Whether they have any role in the process of cancer induction is unknown. Running parallel with this work on the transformation and elimination of carcinogens, the work of White and White (16) has shown that certain of these agents fed in appropriate doses to young rats cause growth failure analogous to that induced by many substances known to be removed as mercapturates. Unless the assumption is made that the oxidation products cause growth inhibition prior to their elimination, the natural conclusion is that the carcinogens are partially detoxicated by mechanisms involving the S-containing amino-acids, though no mercapturate or other S-containing end products have, as yet, been detected.

Interference with sulphur metabolism may, on the basis of the work of White and White, also account for the findings of Badger, Elson, Haddow, Hewett, and Robinson (1), who showed that the intraperitoneal administration of colloidal carcinogens, and some chemically related noncancerogenic compounds, definitely inhibited the growth of transplanted, chemically induced, and spontaneous tumors. This growth inhibition did not appear to be specific for tumors, since general body growth was also retarded.

All the work mentioned above is concerned with mechanisms of elimination of carcinogens, and the biological effects can be visualized as secondary to sulphur deprivation, though precise proof is lacking. The part played by sulphur in the carcinogenic process, if any, still remains an open question.

In an attempt to understand the effect of inhibitors of sulphur metabolism on cancer induction, two points of view may be considered:

1. The association of growth with sulphur metabolism is well established. On this basis it could be assumed that local S-depletion lowers the potentiality for normal growth and this, in turn, delays or prevents the incidence of abnormal growth; i.e., that the carcinogenic change can occur only in growing cells. As an extension of this, if it be supposed that the carcinogenic change is unaffected by S-depletion and occurs within normal time limits, then the apparent inhibition could be attributed to the delay in the rate of growth of malignant cells. This conception of the action of inhibitors of sulphur metabolism would certainly account for the delayed tumor emergence shown in Fig. 1. It implies that they are entirely unspecific and that they could well be replaced by any substances that depress the level of any of the chemical factors indispensable for cellular growth.

2. A consideration of the results shown in Fig. 2 suggests that this conception is inadequate. The distinc-
tion between growth, whether normal or malignant, and the processes culminating in carcinogenic change must be emphasized. When the application of a carcinogen to a precancerous tissue is stopped, tumors continue to appear without further stimulus in numbers determined by the intensity of the pretreatment. The inference is that the limited treatment has either already induced the carcinogenic change or that the metabolic conditions prevail that inevitably lead to this event.

In either case the action of an inhibitor of sulphur metabolism that solely interfered with growth could only retard the emergence of visible warts, and its removal should permit their normal development. In the experiment shown in Fig. 2 this occurred in a few cases, but the majority of the mice remained tumor-free for 6 months after the bromobenzene treatment was discontinued and growth was therefore not impeded. To account for such results the concept of reversibility must be introduced, a reversibility made possible by occasional interferences with sulphur metabolism. This result would be anticipated on the hypothesis that the carcinogen acts primarily by forming a dissociable complex with cell constituents through sulphur linkages. Wood and Fieser (17), on the basis of considerations of the chemical reactivity of the carcinogen to a precancerous tissue is stopped, tumors continue to appear without further stimulus in numbers determined by the intensity of the pretreatment. The inference is that the limited treatment has either already induced the carcinogenic change or that the metabolic conditions prevail that inevitably lead to this event.

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SUMMARY AND CONCLUSIONS

The carcinogenic action of 3,4-benzpyrene on mouse skin is inhibited, and sometimes prevented, by local applications of bromobenzene to the skin 4 times weekly.

In a precancerous area of skin, bromobenzene greatly delays, and often prevents, the emergence of visible tumors.

The influence of bromobenzene is local and evidence is given that it is due to intermittent interference with sulphur metabolism. Glutathione and ascorbic acid levels in the skin are lowered quickly after bromobenzene treatment, but recover to normal values after a few hours. All the chemical and biological evidence supports the view that bromobenzene is detoxicated by mercapturate formation in the skin before being excreted in the urine.

The possible relation of sulphur metabolism to carcinogenesis is discussed.

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